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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Method for the Targetted Change of a Gene

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(57) 4 Claims

Abstract

For the targetted change of a gene within the genome of intact mammalian cells by homologous recombination a DNA sequence, which is homologous to the gene to be changed but, however, differs by at least one nucleotide from its sequence as a result of mutation, deletion, or insertion, is introduced into appropriate cells by microinjection and cells are isolated in which the resultant change can be detected.

Description

The invention concerns a method for the targetted change of a gene within the genome of intact mammalian cells by homologous recombination.

The investigation of the causes of different genetic diseases and the localization of the defects at the molecular level which produce the disease is progressing with increasing rapidity thanks to the application of genetic engineering which is becomming more precise and reliable. Nevertheless, the function of many genes of interest, particularly in mammalian cells, has not yet been elucidated because, inter alia, no satisfactory methodology yet exists for the mutagenesis of intact mammalian cells. Moreover, the same methodology would be necessary to correct genetic defects which are already known.

Up to now, in first attempts at this in mammalian cells, when a defective gene is present attempts have been made to replace its function by insertion of a corresponding functional gene at another site, however, in this process other changes which are active over long periods may be caused at a site other than that which is to be mutated which is why this method is ruled out for a possible treatment of disease in mammalian cells.

Another major disadvantage of the experimental methods up to now for mutagenesis by homologous recombination in mammalian cells is that the introduction of a marker gene is necessary to detect the resultant mutation. These marker genes, which are foreign genes at this site or, in general, in the genome, must also be expected to

result in undesired side effects with a high degree of probability.

In addition, the success rate of the methods used up to now for in vivo mutagenesis, also known as "gene targetting" is relatively low in mammalian cells.

It is therefore one of the major aims for the further development of genetic engineering and also the object of the present invention to provide a method which enables the direct alteration of a specific part of the mammalian cell genome in living cells without at the same time involving the risk that other unintentional mutations are caused or that foreign sequences have to be inevitably introduced.

This object is achieved according to the present invention by a method for the targetted change of a gene within the genome of intact mammalian cells by homologous recombination, in which a DNA sequence, which is homologous to the gene to be changed but, however, differs by at least one nucleotide from its sequence as a result of mutation, deletion, or insertion, is introduced into appropriate cells by microinjection and those cells are isolated in which the resultant change can be detected.

The method according to the present invention results surprisingly in a particularly large proportion of homologous recombinations at the desired sites whereby a homologous recombination occurred in more than one in 640 cells. Even a recombination rate of 1 per 150 microinjected cells could already be achieved with the method according to the present invention. This is an

exceptional increase in the efficiency compared to the relation of homologous to illegitimate recombinations in mammalian cells using previous methods. Furthermore, the resultant change can be detected in the method according to the present invention without having to introduce a marker gene and without a corresponding selection by means of this marker gene. The change can be detected after cloning the microinjected cells by Southern blotting or also sequencing.

In a particularly preferred embodiment of the invention the detection of the resultant change is carried out using the PCR (polymerase chain reaction) method (R.K. Saiki et al., Science 239, (1988) 487-491). Using this, it can be established whether the desired mutation has taken place without prior cloning of the cells. In this case the method is so sensitive that even a few DNA molecules can be reliably analyzed without the need for a large number of cells. The method can even be applied in such a way that point mutations are detectable.

Two primers are used for the detection, one of which is complementary to the position of the DNA sequence differing from the gene. An amplification from this primer can only occur and be detected when a recombination has taken place.

Every natural DNA sequence which has been isolated and, if desired, cloned or also DNA sequences produced synthetically can be used as the DNA sequence which is homologous to the gene to be changed but which differs in at least one position from the sequence of this gene. In this process one has assumed up to now that the length of the homology is directly proportional to the frequency of the occurrence of the gene conversion

whereas the length of the heterology is inversely proportional to it. It is therefore particularly preferable to use a DNA sequence which only differs from the genomic sequence to be changed at the required positions but which has as large a homology as possible from which it can be concluded that the rate of recombination is higher the longer the DNA sequence used.

In a preferred embodiment of the invention somatic stem cells are used as mammalian cells. Previous knowledge of such stem cells and gene transfer into such cells is summarized for example by P.M. Dexter and D. Boettinger, Gene Transfer into Hemapoetic Cells by Retroviral Vectors, ISI, Atlas of Science, 167-174. After introduction of the cells into a mammal, the change according to the present invention of the genome of somatic stem cells enables an existing and known defect to be rectified since the stem cells differentiate to functional cells and defective populations in the body are replaced successively. Blood defects such as factor VIII deficiency (haemophilia) are examples of the application for the correction of defective genes by (re-)introduction of mutated stem cells according to the present invention. A further possibility for an application would result if the described defects in the insulin gene could be corrected in stem cells which differentiate to cells of the islet of Langerhans so that here there is a chance to combat diabetes at a genetic level. Somatic cells are either kept in culture on a so-called "feeder cell" layer e.g. a layer of mitotically inactive cells incapable of division or which have been prevented from differentiating with the aid of growth factors (DIA: A.G. Smith et al., Nature,

Vol. 336, (1988), Page 688, LIF: R.L. Williams et al., Nature 336 (1988), page 684) and thus enable a culture.

Applications of the method according to the present invention which for example are directed at increasing the secretion of growth factors in animals appear of interest for agriculture and in particular for the breeding of useful animals whereby in animal breeding the mutated gene can then be introduced into the germ track for instance by injection of zygotes into a fertilized egg or by use of embryonic stem cells.

A further important possibility for an application of the method according to the present invention using somatic or embryonic stem cells and, if desired, the injection of zygotes into a fertilized egg, is the opportunity which thus presents itself to investigate the function of hitherto unknown genes in animal models. Thus, for example, stem cells from mice, which contain a gene mutated at a particular site can be injected into a fertilized egg and the effects on a chimeric animal obtained in this way can be examined. A further exceptionally important possibility for an application comprises the introduction of cells mutated according to the present invention into the germ track of animals such as e.g. mice to generate a mouse model for diseases diagnosed in humans. By this means it would be possible to investigate the effect of pharmaceutical agents in a mouse model in which this mouse model would be very meaningful for the application in man.

For the future the method according to the present invention also appears to offer a possibility to cultivate improved or changed e.g. resistant plants. For this purp se the same method can, in principle, be

applied as for mammalian cells as soon as a means has been created for the efficient introduction of DNA into such plant cells by microinjection and for a homologous recombination.

In summary it is possible by means of the method according to the present invention in a hitherto unexpected and surprising manner to carry out a change at the target site in living cells and not at a homologous site, and to carry out a correction in somatic gene therapy in which no additional foreign DNA sequences whatsoever are introduced into the cells and no long-term defects need to be feared.

The following Examples in conjunction with the Figures elucidate the invention further.

Fig. 1 shows a diagram of the modification of the Hox 1.1 gene;

Fig. 2 shows a Southern analysis of the genomic DNA amplified by PCR from microinjected 3T3- (a) and D3-cell pools (b) and a diagram of the PCR strategy (c);

Fig. 3 shows a Southern analysis of genomic DNA from cloned embryonic stem cells (a, b, c) digested by KpnI and a diagram of the mutated and the normal Hox 1.1 allele as well as the expected restriction fragments; and

Fig. 4 shows a Southern blot of the DNA amplified by PCR from tail biopses from two litters of genetically altered mice.

E x a m p l e 1

Mutagenesis of the Hox 1.1 gene.

A FspI fragment of the Hox 1.1 gene (nucleotide 367 to 1937, numbered from the start codon (see Fig. 1) (A.M. Colberg-Poley et al., Nature 314 (1985), 713-717, M. Kessel et al., Proc. Natl. Acad. Sci. USA 84 (1987) 5306-5310) was cloned in Bluescript (Stratagene) and a 20 bp oligonucleotide was inserted into the EcoRI site of the homeobox. The sequence of the inserted oligonucleotide was AAT TGT GAG GTA CCG CTG AC. This construct shown in Fig. 1, line 2 was microinjected in an injection volume of 10^{-11} ml by microinjection into the nucleus of NIH-3T3 cells (200 to 600 cells) or D3 cells (T.C. Doetschmann et al., J. Embryol. exp. Morph. 87 (1985) 27-45) (50 to 200 cells) at a concentration of 300 ng/ml which corresponds to 5 molecules of DNA per cell. After the microinjection the cells were allowed to grow for 4 to 7 days, trypsinized and applied again onto the same plate. The cells were collected again after 4 to 7 days. Half of the cells were used for DNA analysis, the remainder was stored in liquid nitrogen. The genomic DNA or a control plasmid (1 µg or 15 pg) were subjected to 30 PCR cycles using heat-stabilized taq polymerase (Saiki, R.K. et al., Science 239, 487-491 (1988), Letson, A. and Liskay, R.M., Genetics, 117, 1987, 759-769) in a 50 µl reaction mixture containing 10 % dimethylsulphoxide, 67 mM Tris-HCl (pH 8.8), 16.6 mM NH_4SO_4, 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 170 µg/ml bovine serum albumin (BSA), 450 µM of each of the four deoxyribonucleoside triphosphates (dATP, dCTP, dTTP, dGTP) and 0.5 µM of two primers. The first primer was a synthetic oligonucleotide with the sequence TTC CGC ATC TCA CCC TGG AT, which is specific for a sequence in the

first ex n of the Hox 1.1 gene and which binds on the 5' side of the Fspl cleavage site, and the second primer has an identical DNA sequence to the above-mentioned synthetic oligonucleotide. The samples were overlayered with paraffin and heated in each cycle to 91.5°C for one minute in order to denature the DNA, cooled to 55°C for one minute in order to allow the primer to bind and heated to 70°C for 6 minutes to activate the polymerase. Since the normal Hox 1.1 allele contains no binding site for the second primer and the microinjected fragment contains no binding site for the first primer, the sought-after fragment can only be amplified by the polymerase chain reaction in cells mutated according to the present invention. Aliquots of the reaction mixture (10 µl) were subjected to electrophoresis on a 1 % agarose gel, transferred onto Gene Screen Plus (DuPont) and examined in 50 % formamide, 1 M NaCl and 1 % SDS with a hybridization probe which was labelled with ^{32}P to an activity of $1x10^9$ cpm/µg using a Multiprime kit (Amersham) and used with $5x10^5$ cpm/ml. The filters were washed in 2xSSC, then in 2xSSC-1 % SDS and finally in 0.1xSSC (1xSSC: 8.765 g/l NaCl, 4.41 g/l sodium citrate, pH 7.0) at 65°C. Fig. 2 shows an autoradiogram of the dried filters; part a after 2 hours and part b after 5 hours exposure. In this connection, Fig. 2a shows a Southern analysis of genomic DNA amplified by PCR from microinjected 3T3 pools (tracks 1 to 3) and of the plasmid pH 1.1/O-BF in track 4 as control. This plasmid contains the entire Hox 1.1 gene and the inserted oligonucleotide. In part b of the diagram a Southern analysis of genomic DNA from microinjected D3 cell pools amplified by PCR is shown. Fig. 2c shows a diagram of the plasmid pH1.1/O-BF. A 3643 base pair long 5-BamHI-FspI fragment of the Hox 1.1 gene (nucleotide - 1711 to + 1932 in relation to the translation start codon) was cloned in Bluescript (Stratagene) and the intervening

oligonucleotide was cloned in the EcoRI site of the homeobox.

E x a m p l e 2

Further investigation of the stem cells

Two cell pools of the embryonic stem (ES) cells D3 from Example 1 (G.R. Dressler and P. Gruß, Trans Genet. 4, 214-219 (1988), T.C. Doetschmann et al., J. Embryol. Exp. Morph. 87 (1985), 27-45) were used for the further analysis. Single cell clones from these pools were picked out by drawing up individual cells with glass capillaries (M.A. Rudnicki & M.W. McBurney in Teratocarcinomas and Embryonic Stem Cells, (ed. E.J. Robertson) 19-49 (Publisher, Town, 1987). Clonal cell lines were prepared from these cells by plating first on plates with 96 wells, then on plates with 24 wells of 1.5 cm diameter and then in 6 cm tissue culture plates. DNA was isolated from half of these cell lines; the other half was stored in liquid nitrogen. During the entire cloning procedure the ES cells were kept in culture on so-called "feeder cells". Aliquots (10 µg) of the genomic DNA were digested with KpnI, subjected to electrophoresis on 0.8 % agarose gels and transferred to Gene Screen Plus. The filters were hybridized with ^{32}P-labelled probes as described in Example 1 and washed as described there. Since the inserted oligonucleotide has a KpnI restriction site, a new 5.4 kb band was expected to occurr in homologous recombinant clones in addition to the 14 kb band of the normal allele after hybridization with a probe (probe 1) specific for the first exon in Fig. 2d. The occurrence of this band is shown in track 1 of Fig. 2a. The ES cell DNA was contaminated with feeder cell DNA because the ES cells

were prevented from forming large colonies during the sub-cloning. Therefore the 14 kb Hox 1.1 fragment not only represents the wild-type allele from ES cells but also wild-type alleles from the feeder cells. Altogether 161 individual clones were analyzed by Southern blotting from pool 1 and 165 clones from pool 7. In order to confirm and to enlarge on the Southern analysis clones from both pools were cultured until large ES colonies formed on the feeder cells. The relative amounts of ES cell DNA were then very much larger as can be seen from Fig. 3b and c. A Southern blot of DNA digested with KpnI from one of the clonal lines derived from pool 1 was hybridized with probe 1 (Fig. 3b, line 1) and probe 2 (Fig. 3b, track 2). Details of the probes are shown in Fig. 3c. The relative intensities of the 5.4 kb and the 8.6 kb bands correspond to the 5' and 3' fragments (see Fig. 3d) in comparison with that of the 14 kb fragment which shows that at least 50 % of the ES cells contain the mutated allele. DNA from a clonal line from pool 7 was digested with StuI/KpnI (Fig. 3c, track 1) and KpnI (Fig. 3c, track 2) and Southern blots were hybridized in succession with probe 3 (see Fig. 3d). The 600 bp and 4.27 kb fragments (Fig. 3c, track 1) which form are derived from mutated and normal alleles (see Fig. 3 for further details). The mutated and the normal allele are present in the same amounts in the StuI/KpnI and the KpnI digestion mixture (Fig. 3c, tracks 1 and 2) which shows that the ES cells from pool 7 are clonal. An analysis of the karyotype of the clone derived from pool 1 showed that 70 % of the cells contained 40 chromosomes. It is therefore assumed that the low frequency of occurrence of the mutated allele in this cell line is a consequence of contamination by non-mutated ES cells and does not indicate a chromosomal instability. It was estimated that the ratio of homologous to illegitimate recombinations is 1:30 since

about 20 % of the microinjected cells were stably transformed after nuclear microinjection (M.P. Capecchi, Cell 22 (1980) 479-488). The homologous recombination took place in 1 out of 150 microinjected cells. This is surprisingly high compared to comparable investigations (K.R. Thomas & M.R. Capecchi, Cell 51 (1987) 503-512, O. Smithies et al., Nature 317 (1985) 230-234, K.R. Thomas et al., Cell 44 (1986), 419-428).

E x a m p l e 3

The embryonic stem cell lines obtained in Example 1 and shown in Fig. 3a and 3b (litter 1: tracks 1 to 4 of Fig. 4, litter 2: tracks 5 to 7 of Fig. 4) were introduced into blastocytes of C57BL/6 mice and two litters were obtained with a total number of 4 chimeric animals. In order to confirm that the chimeric animals contain the mutated allele a biopsy was taken from the tails of the animals and 100 ng of genomic DNA from these biopsies was subjected to 30 PCR cycles as described in Example 1. The tracks 2, 5, 6 and 7 of Fig. 4 show an amplification of the 1.1 kb fragment which exhibits the mutated allele (see Fig. 1 and Fig. 2).

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:-

1. Method for the targetted change of a gene within the genome of intact mammalian cells by homologous recombination, w h e r e i n a DNA sequence, which is homologous to the gene to be changed but, however, differs by at least one nucleotide from its sequence as a result of mutation, deletion, or insertion, is introduced into appropriate cells by microinjection and cells are isolated in which the resultant change can be detected.

2. Method as claimed in claim 1, w h e r e i n somatic stem cells are used as the mammalian cells.

3. Method as claimed in claim 1 or 2, w h e r e i n a PCR reaction is carried out to detect the resultant change.

4. Method as claimed in one of the claims 1 to 3, w h e r e i n the gene to be changed is a gene which causes symptoms of disease and a DNA sequence is used which corresponds to the healthy gene.

FIG.1

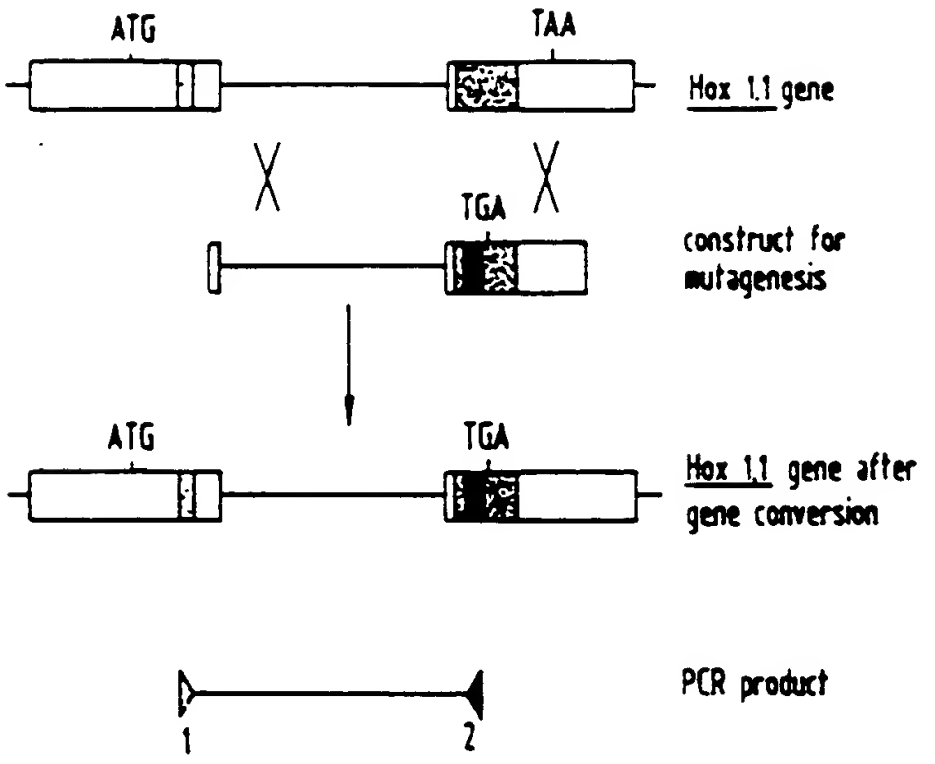
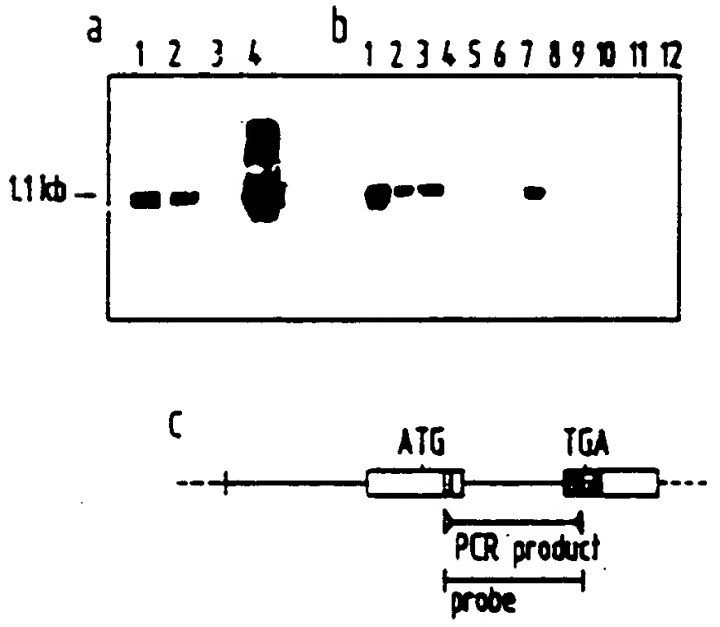


FIG.2



PATENT AGENTS

Dunlop Ogilvy Renault

FIG.3

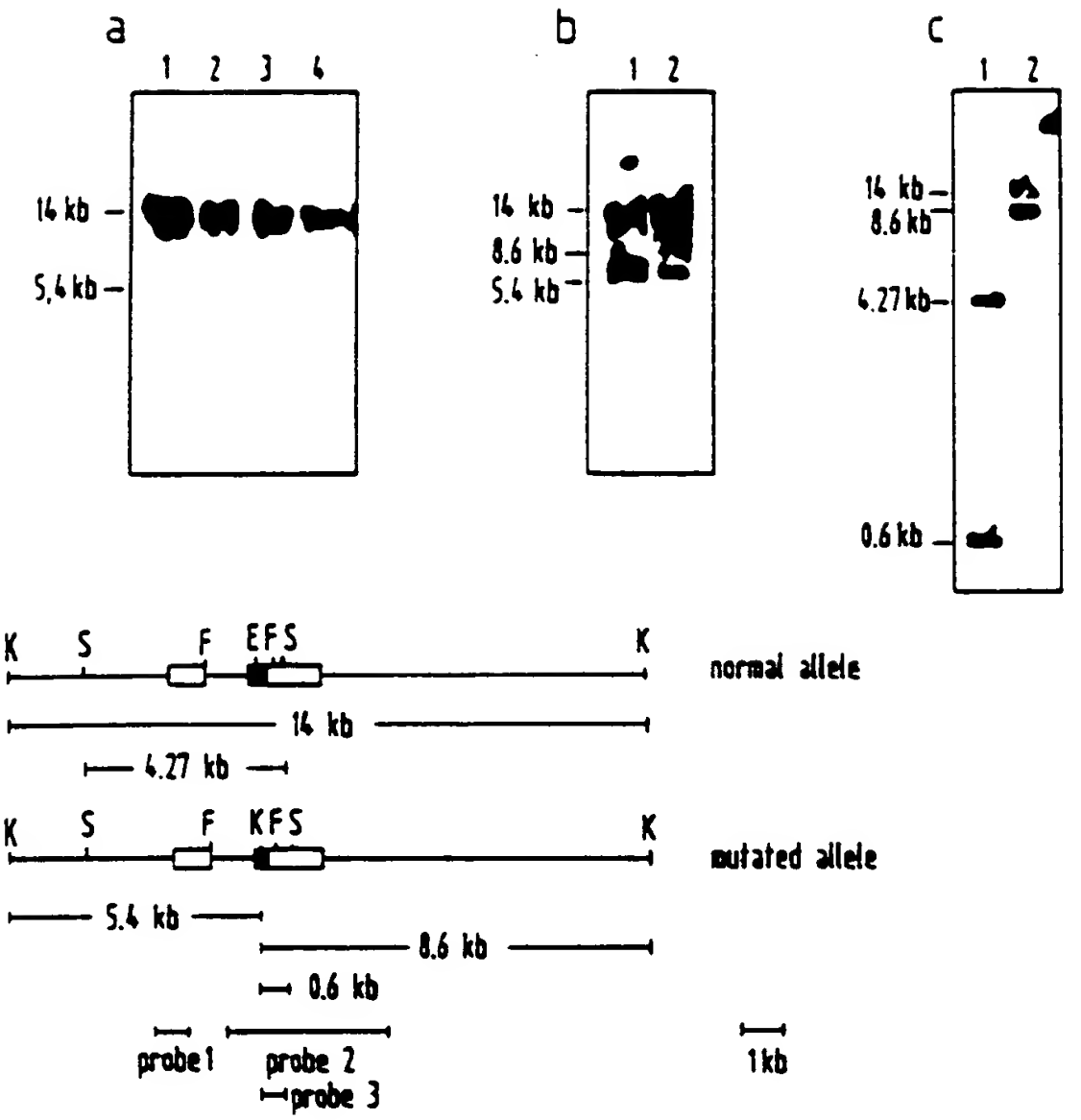
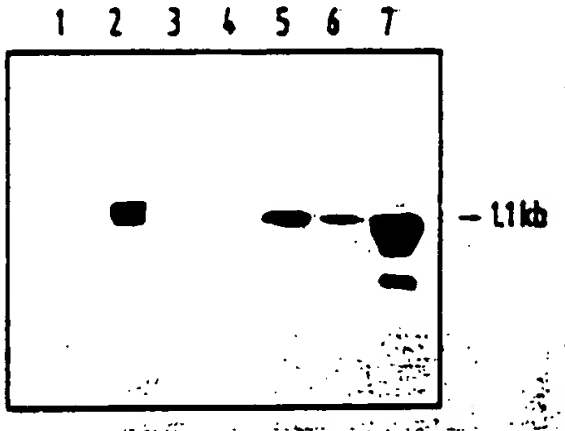


FIG.4



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Beresford Ogilvy Renault